Calcium-Independent Phospholipase A_2 Mediates Proliferation of Human Promonocytic U937 Cells

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Abbreviations: AA, arachidonic acid; BEL, bromoenol lactone; MAFP, methyl arachidonyl fluoromethyl phosphonate; PLA_2, phospholipase A_2; $iPLA_2$-VIA, group VIA calcium-independent phospholipase A_2; $cPLA_2\alpha$, group IVA cytosolic phospholipase A_2; PC, phosphatidylcholine.

Running Title: $iPLA_2$-VIA Mediates U937 Cell Proliferation

ABSTRACT

We have investigated the possible involvement of two intracellular phospholipase A_2s, namely Group VIA calcium-independent phospholipase A_2 (iPLA$_2$-VIA) and Group IVA cytosolic phospholipase A_2 (cPLA$_2\alpha$), in the regulation of human promonocytic U937 cell proliferation. Inhibition of iPLA$_2$-VIA activity by either pharmacological inhibitors such as bromoenol lactone or methyl arachidonyl fluorophosphonate, or specific antisense technology strongly blunted U937 cell proliferation. In contrast, inhibition of cPLA$_2\alpha$ had no significant effect on U937 proliferation. Evaluation of iPLA$_2$-VIA activity in cell **cycle-synchronized cells** revealed highest activity at G_2/M and late S phases, and lowest at G_1. PC levels showed the opposite trend, peaking at G_1 and being lowest at G_2/M and late S. Reduction of U937 cell proliferation by inhibition of iPLA$_2$-VIA activity was associated with arrest in G_2/M and S phases. The iPLA$_2$-VIA effects were found to be independent of the generation of free arachidonic acid or of one of its oxygenated metabolites, **and may work through regulation of the cellular level of PC, a structural lipid which is required for cell growth/membrane expansion.**

Key words: phospholipase A_2; cell cycle; proliferation; membrane phospholipid; human promonocytes.

The phospholipase A_2 (PLA_2) superfamily constitutes a heterogeneous group of enzymes with distinct roles in cell function [1-5]. The common feature of these enzymes is that they all selectively hydrolyze the fatty acid at the sn-2 position of glycerophospholipids. However, it is becoming increasingly clear that PLA_2s notably differ in substrate specificity, cofactor requirements for activity, and cellular localization [1-5]. Mammalian cells usually contain several PLA_2s, and thus the challenge in recent years has been that of ascribing specific cellular functions to particular PLA_2 forms. PLA_2s are systematically classified into several group types, with many of them including various subgroups [5]. However, attending to biochemical commonalities, PLA_2s are usually grouped into four major families, namely the Ca^{2+}-dependent secreted enzymes, the Ca^{2+}-dependent cytosolic enzymes ($cPLA_2$), the Ca^{2+}-independent cytosolic enzymes ($iPLA_2$), and the platelet-activating factor acetyl hydrolases [1, 5].

The $iPLA_2$ family consists of two members in mammalian cells, designated $iPLA_2$-VIA and iPLA2-VIB, of which the former is the best characterized [3,6,7]. Since its purification [8] and cloning [9, 10] in the mid nineties, $iPLA_2$-VIA has attracted considerable interest due to the multiple roles and functions that this enzyme may serve in cells. Several splice variants of $iPLA_2$-VIA co-exist in cells and thus it is conceivable that multiple regulation mechanisms exist for this enzyme, which may depend on cell type. Thus, $iPLA_2$-VIA PLA_2 may be a multi-faceted enzyme with multiple functions of various kinds (i.e. homeostatic, catabolic, and signaling) in different cells and tissues [3, 7].

Several lines of evidence have suggested a key role for $iPLA_2$-VIA in the control of the levels of phosphatidylcholine (PC) in cells by regulating basal deacylation/reacylation reactions. This is manifested by the significant reduction of the steady-state level of lysoPC that is

observed shortly after acute inhibition of iPLA$_2$-VIA by cell treatment with bromoenol lactone (BEL) [11, 12]. In INS-1 insulinoma cells, acute inhibition of iPLA$_2$-VIA reduces the relatively high content of lysoPC that these cells exhibit by about 25% [12], whereas in macrophage-like cell lines, the decrease is about 50-60% [11, 13, 14], suggesting that the dependence of PC metabolism on iPLA$_2$-VIA may vary from cell to cell. In some cell types, particularly -but not uniquely- phagocytes [11, 13-19], reduction of the steady-state level of lysoPC slows the initial rate of incorporation of exogenous arachidonic acid (AA) into cellular phospholipids. In other studies, it has been shown that iPLA$_2$-VIA may be coordinately regulated with CTP:phosphocholine cytidylyltransferase to maintain PC levels [20-23]. Given that PC is the major cellular glycerophospholipid present in mammalian cell membranes and thus serves a key structural role, we hypothesized that iPLA$_2$-VIA may play an important role in processes such as cell proliferation where membrane phospholipid biogenesis has to occur. Thus we studied the possible involvement of iPLA$_2$-VIA in the normal proliferative response of human promonocytic U937 cells, and compared it to that of another major intracellular PLA$_2$, the AA-selective cPLA$_2\alpha$. Utilizing different strategies, we demonstrate here that iPLA$_2$-VIA, but not cPLA$_2\alpha$, plays a key role in U937 cell proliferation by a mechanism that does not involve AA or one of its oxygenated metabolites.

RESULTS

iPLA$_2$ inhibition slows U937 cell proliferation - Using RT-PCR, we have previously found that human promonocytic U937 cells express both cPLA$_2\alpha$ and iPLA$_2$-VIA but, strikingly, do not express Group V, Group X or any of the Group II sPLA$_2$s [24]. Enzymatic activities corresponding to both cPLA$_2\alpha$ and iPLA$_2$-VIA can be readily detected in the U937 cells by utilizing specific enzyme assays and inhibitors [18, 25]. We began the current study by

investigating whether the activities of these two intracellular phospholipases are required for normal U937 cell growth (i.e. that induced by the serum present in the culture medium, in the absence of any other mitogenic stimulus). In the first place, the effect of various selective PLA_2 inhibitors was examined. Fig. 1 shows that the selective $cPLA_2\alpha$ inhibitor pyrrophenone **[26]** completely blocked the Ca^{2+}-dependent PLA_2 activity of U937 cell homogenates at concentrations as low as 0.5-1 μM. However, at these concentrations, pyrrophenone failed to exert any effect on the proliferation of U937 cells, as measured by a colorimetric staining assay (Fig. 1).

In contrast to pyrrophenone, the $iPLA_2$ inhibitor BEL strongly blocked the growth of the U937 cells (Fig. 2). In these experiments, a BEL concentration of 5 μM was utilized to avoid the proapoptotic effect of this drug when used at higher concentrations [27-29]. We confirmed that at 5 μM, BEL significantly blunted cellular $iPLA_2$ activity, as measured by an in vitro assay (Fig. 2). Collectively, the data in Figs. 1 and 2 are consistent with the involvement of $iPLA_2$, but not $cPLA_2\alpha$, on U937 cell proliferation. Owing to the lack of specificity of BEL in cell-based assays [28], additional pharmacological evidence for the involvement of $iPLA_2$ in U937 cell growth was obtained by using MAFP, a dual $iPLA_2$/$cPLA_2$ inhibitor that is structurally unrelated to BEL and pyrrophenone [30, 31]. Concentrations of MAFP that completely inhibited cellular Ca^{2+}-independent PLA_2 activity also led to strong inhibition of U937 cell growth (Fig. 3). Given that the pyrrophenone experiments established that $cPLA_2\alpha$ is not critical for U937 cell growth, the inhibitory effect of MAFP seen in Fig. 3 can be attributed to inhibition of $iPLA_2$.

To confirm $iPLA_2$ involvement in U937 cell growth in a more definitive manner, the effect of an antisense oligonucleotide directed against $iPLA_2$-VIA was evaluated. In these

experiments, this antisense produced a 70-75% decrease of both immunoreactive iPLA$_2$-VIA, and markedly inhibited **(30-40%)** U937 cell proliferation (Fig. 4).

Inhibition of iPLA$_2$ does not induce cell death - Trypan blue assays after the different treatments leading to iPLA$_2$ inhibition indicated no loss of viability, thus suggesting that necrotic cell death did not occur. To examine the possibility of apoptotic cell death, we utilized the annexin V-binding assay, which measures externalization of phosphatidylserine, a marker of apoptosis. Incubation of the U937 cells with 10 μM MAFP or 5 μM BEL **for 24 h**, conditions that result in inhibition of iPLA$_2$ activity and cell growth as shown above (Figs. 2 and 3), had no effect on the number of annexin V-positive cells, which always remained below 12% of the total cell population. Antisense inhibition of iPLA$_2$-VIA also did not increase the number of annexin V-positive cells. As a control for these experiments, we also studied the effect of a higher BEL concentration, i.e. 25 μM, which is known to induce apoptotic cell death in U937 cells in an iPLA$_2$-independent manner [27]. Confirming our previous data, 25 μM BEL increased the extent of apoptotic cell death well above 75% **after a 24-h incubation period**. Together, these data indicated that the slowed growth of cells deficient in iPLA$_2$ activity by either pharmacological or antisense means arises as a result of slowed cell division and not of increased apoptosis.

iPLA$_2$ Activity During the Cell Cycle - To gain more information on the role of iPLA$_2$ on U937 cell growth, we used flow cytometry to examine the cell cycle dependence of iPLA$_2$ activity in the U937 cells. The cells were synchronized with nocodazole [23, 32] and then allowed to progress through the cell cycle under normal culture conditions. Immediately after release from the mitotic block with nocodazole, more than 75% of the cells were in G_2/M (Fig. 5). The cells were in G_1 from 2 to 8 h after release from nocodazole, and in S thereafter

up to 10 h. After 10 h the cells became largely asynchronous again. Thus, this method allows study of the cell cycle of U937 cells in G_2/M throughout G_1 and S phases [23, 32].

iPLA$_2$ activity measurements along the cell cycle revealed significant differences depending on the phase where the cells were (Fig. 5). Highest activity was found during G_2/M, decreasing as the cells entered G_1 and then increasing again as the cells approached and entered S phase. Of note, the same pattern of variation of iPLA$_2$ activity along the cell cycle was detected whether the assay was conducted with mixed micelles, vesicles, or natural membranes as substrates (not shown), thus confirming the biological relevance of the findings. Importantly, quantification of the levels of PC, the major membrane phospholipid in mammalian cells, during the cell cycle showed a pattern that was clearly opposite to that found for iPLA$_2$ activity (Fig. 5). PC levels rose abruptly in early G_1 and then slowly declined as the cells were progressing into late G_1 and S (Fig. 5). That PC mass and iPLA$_2$ activity show opposite kinetics is fully consistent with the possibility that iPLA$_2$ behaves as a major regulator of PC catabolism, which is responsible for glycerophospholipid accumulation during the cell cycle [23, 33]. Thus, decreased iPLA$_2$ activity during phase G_1 would result in an increase in PC mass due to reduced catabolism.

Induction of cell cycle arrest by iPLA$_2$ inhibition - Having established that iPLA$_2$ activity is cell-cycle regulated, the levels of which inversely correlate with those of the major membrane phospholipid PC, we set out to investigate next whether the slowed growth due to iPLA$_2$ inhibition was a consequence of cell cycle arrest. The cells were synchronized with nocodazole and then treated with BEL to inhibit iPLA$_2$ activity. Pyrrophenone was also used as a control. Fig. 6 shows that treatment of the cells with BEL induced a significant accumulation of cells in G_2/M and S, and a concomitant decrease of cells in G_1, with respect to untreated cells. Pyrrophenone, on the contrary, induced no significant changes in the phase

distribution (Fig. 6). Thus these data suggest that inhibition of $iPLA_2$, but not $cPLA_2\alpha$, causes cell arrest in S and G_2/M phases.

Arachidonic acid and/or its metabolites are not involved in U937 cell growth - In addition to its role in PC homeostasis, $iPLA_2$, as a sn-2 lipase, may also participate in generating free fatty acids such as AA, which could subsequently be metabolized to eicosanoids. The importance of AA and the eicosanoids as growth factors towards various cell types has previously been demonstrated [34]. We tested first the effects of various cyclooxygenase and lipoxygenase inhibitors on the growth of U937 cells under normal culture conditions. The inhibitors employed were aspirin (up to 25 μM), indomethacin (up to 25 μM), NS-398 (up to 10 μM), ebselen (up to 10 μM), baicalein (up to 10 μM), MK-886 (up to 10 μM), and nordihydroguaiaretic acid (up to 10 μM). Control experiments had indicated that at the concentrations employed, these inhibitors effectively blocked AA oxygenation by the cyclooxygenase and lipoxygenase pathways. None of the inhibitors exerted any significant effect on U937 cell growth (data not shown). We studied next whether adding 10 μM AA to the cell cultures attenuates the antiproliferative effect of inhibiting $iPLA_2$ by BEL or antisense technology. The results indicated however that AA failed to restore the growth of cells deficient in $iPLA_2$ activity. Moreover, when the cells were synchronized with nocodazole, the subsequent addition of exogenous AA exerted no detectable effect on the observed phase distribution (see Fig. 6), whether the cells had been treated or not with BEL (not shown). Collectively, these results suggest that AA or a metabolite does not mediate the effect of $iPLA_2$ on U937 cell proliferation.

DISCUSSION

In this study we demonstrate that $iPLA_2$-VIA is required for the proliferation of human

promonocytic U937 cells under normal culture conditions (i.e. in the absence of any mitogenic stimulus other than serum), and that inhibition of iPLA$_2$-VIA by either pharmacological means or antisense technology slows growth by inducing arrest at S and G_2/M phases. Cell accumulation at these phases of the cell cycle could not be reversed by supplying the medium with exogenous AA, indicating that the iPLA$_2$-VIA role is not mediated via AA-derived mitogenic signaling. We also document that U937 cell iPLA$_2$-VIA activity is regulated in a cell cycle dependent manner, with maximal activity at G_2/M, steadily declining during G_1, and rising up at late S phase. Strikingly, the levels of PC, the major membrane phospholipid in mammalian membranes, exhibit the opposite kinetics, with the highest levels at G_1. This inverse relationship between the kinetics of iPLA$_2$-VIA activity and PC accumulation agrees with previous studies in Jurkat cells [32] and CHO-K1 cells [23]. It is well established that changes in PC content during the cell cycle correlate better with the kinetics of its catabolism rather than synthesis [23, 33, 35], and the involvement of iPLA$_2$-VIA in the homeostatic regulation of membrane phospholipid turnover is one of the first roles attributed to this enzyme in cells [6, 7]. Thus our results are in line with a scenario whereby iPLA$_2$-VIA plays a central role in cell growth and division by regulating glycerophospholipid metabolism during the cell cycle [23, 32, 36]. Thus, down-regulation of iPLA$_2$-VIA activity in G_1 and early S would allow for accumulation of phospholipid in preparation for future cell division. Once cells enter S phase, iPLA$_2$-VIA begins to increase, which would slow down phospholipid accumulation. **It is interesting to note, however, that iPLA$_2$-VIA may not always be the major regulator of phospholipid catabolism during cell cycle progression. Our data suggest that two hours after cell entry, iPLA$_2$ is drastically reduced but PC levels appear to barely change (Fig. 5), raising the possibility that, at this time, involvement of fatty acid-reacylating enzyme activities or inter-phospholipid/diacylglycerol transacylation might be significant in regulating PC levels.**

Whether in addition to regulating glycerophospholipid metabolism during the cell cycle, iPLA$_2$-VIA may also act by activating receptor-mediated mitogenic signaling --e.g. by directly mediating the generation of lipid mediators with growth factor-like properties-- is also a possibility that deserves consideration. Although we and others [23, 32, 37] find no evidence for the involvement of AA and/or its metabolites in regulating cellular proliferation, other studies have reported the involvement of iPLA$_2$ in cell growth via generation of AA, clearly indicating cell type-specific differences. In a recent study Herbert and Walker [38] have described the involvement of iPLA$_2$-VIA in the proliferative response of human umbilical endothelial cells to serum. Inhibition of iPLA$_2$-VIA blocked proliferation, which could be partially restored by supplying the cell cultures with exogenous AA [38]. Similarly, work by Sánchez and Moreno [39] has attributed a key role for iPLA$_2$-VIA-mediated AA release in regulating Caco-2 cell growth.

While our results have excluded the eicosanoids from having effects on cell cycle progression, we do not rule out the possibility that lysophospholipids generated by iPLA$_2$-VIA could be involved in the response. As a matter of fact, iPLA$_2$-VIA has been shown to mediate various responses of monocytes and U937 cells through lysophospholipid generation, namely secretion [10], apoptosis [24, 40, 41] and possibly chemotaxis [42, 43].

The involvement of specific PLA$_2$ forms in the regulation of cell division may also be a cell type-specific event. Although we have failed to implicate cPLA$_2\alpha$ --a well established signal-activated enzyme [34]-- in regulating cellular proliferation, other studies have reported the involvement of this enzyme. In the aforementioned system of human umbilical endothelial cells, the importance of the cPLA$_2\alpha$-mediated AA release in the regulation of cell proliferation has also been documented [44]. Thus the suggestion was made that both enzymes may somehow cooperate in regulating endothelial cell proliferation via generation of

AA [38, 44]. In contrast, the work by Sánchez and Moreno [39] attributed a key role for iPLA$_2$-VIA-mediated AA release in regulating Caco-2 cell growth, but ruled out a role for cPLA$_2\alpha$ in the process. However, studies in vascular smooth muscle cells by Anderson et al. [45] highlighted the very important role of cPLA$_2\alpha$ in the process but the lack of involvement of iPLA$_2$-VIA. Importantly, in a recent study with neuroblastoma cells, van Rossum et al. [46] demonstrated the involvement of cPLA$_2\alpha$ in cell cycle progression and, although in the latter study a role for iPLA$_2$ in this system could not be ascertained, the observation was made that redundancy of functions between cPLA$_2$ and iPLA$_2$ may exist under certain conditions. We are currently performing experiments with different cell systems to study the possibility of cross-talk between cPLA$_2\alpha$ and iPLA$_2$-VIA in cell proliferation, and also to define when and whether the role of iPLA$_2$-VIA in cell growth is to directly generate growth factor-like lipids and/or to regulate changes in overall phospholipid metabolism that could trigger the activation in situ of intracellular mitogenic pathways.

EXPERIMENTAL PROCEDURES

Reagents. [5,6,8,9,11,12,14,15-^{3}H]AA (200 Ci/mmol) was purchased from Amersham Ibérica (Madrid, Spain). Methyl arachidonyl fluorophosphonate (MAFP), bromoenol lactone (BEL), and the human iPLA$_2$-VIA antibody were from Cayman (Ann Arbor, MI, USA). Pyrrophenone was kindly provided by Dr. T. Ono (Shionogi, Japan). All other reagents were from Sigma (St. Louis, MO, USA).

Cell culture. U937 cells were kindly provided by Dr. Patricio Aller (Centro de Investigaciones Biológicas, Madrid, Spain). The cells were maintained in RPMI 1640 medium supplemented with 10% (v/v) fetal calf serum, 2 mM glutamine, penicillin (100

units/ml) and streptomycin (100 μg/ml) [47]. For experiments, the cells were incubated at 37°C in a humidified atmosphere of CO_2/air (1:19) at a cell density of 0.5-1 x 10^6 cells/ml in 12-well plastic culture dishes (Costar).

PLA_2 activity assays. For Ca^{2+}-dependent PLA_2 activity, the mammalian membrane assay described by Diez et al. [48] was used. Briefly, aliquots of U937 cell homogenates were incubated for 1-2 h at 37°C in 100 mM HEPES (pH 7.5) containing 1.3 mM $CaCl_2$ and 100,000 dpm of [^{3}H]AA-labeled U937 cell membrane, used as substrate, **in a final volume of 0.15 ml. Prior to assay, t**he cell membrane substrate was heat at 57°C for 5 min, in order to inactivate coenzyme A-independent transacylase activity [48]. The assay contained 25 μM LY311727 and 25 μM BEL to completely inhibit endogenous secreted and Ca^{2+}-**in**dependent PLA_2 activities **[30, 49-51]**. **After lipid extraction, free [^{3}H]arachidonic acid was separated by thin-layer chromatography, using *n*-hexane/ethyl ether/acetic acid (70:30:1) as a mobile phase [52, 53].** For Ca^{2+}-independent PLA_2 activity, U937 cell aliquots were incubated for 2 h at 37°C in 100 mM Hepes (pH 7.5) containing 5 mM EDTA and 100 μM labeled phospholipid substrate (1-palmitoyl-2-[^{3}H]palmitoyl-glycero-3-phosphocholine, sp. act. 60 Ci/mmol, American Radiolabeled Chemicals, St. Louis, MO) in a final volume of 150 μl. The phospholipid substrate was used in the form of sonicated vesicles in buffer. The reactions were quenched by adding 3.75 volumes of chloroform/methanol (1:2). After lipid extraction, free [^{3}H]palmitic acid was separated by thin-layer chromatography, **using *n*-hexane/ethyl ether/acetic acid (70:30:1) as a mobile phase [52, 53]**. In some experiments $iPLA_2$ activity was also measured utilizing a mixed-micelle substrate or the natural membrane assay. For the mixed micelle assay, Triton X-100 was added to the dried lipid substrate at a molar ratio of 4:1. Buffer was added and the mixed micelles were made by a combination of heating above 40°C, vortexing, and water bath sonication until the solution clarified. The

natural membrane assay was carried out exactly as described above except that $CaCl_2$ was omitted and 5 mM EDTA was added instead. All of these assay conditions have been validated previously for the U937 cell homogenates [18, 25, 54].

Proliferation assay. The CellTiter96 Aqueous One Solution Cell Proliferation assay (Promega) was used, following the manufacturer's instructions. Briefly, cells (10,000 cells per well) were seeded in 96-well plates treated with vehicle or different concentrations of inhibitor. After 24 h, formazan product formation was assayed by recording absorbance at 490 mn with a 96-well plate reader.

Antisense oligonucleotide treatments. The $iPLA_2$-VIA antisense oligonucleotide utilized in this study has been described in previous studies from our laboratory [24, 25, 27, 55]. The antisense or sense oligonucleotides were mixed with lipofectamine, and complexes were allowed to form at room temperature for 10-15 min. The complexes were then added to the cells, and the incubations were allowed to proceed under standard cell culture conditions. The final concentrations of oligonucleotide and lipofectamine were 1 μM and 10 μg/ml, respectively. Oligonucleotide treatment and culture conditions were not toxic for the cells as assessed by the Trypan blue dye exclusion assay.

Immunoblot analyses. Cells were lysed in an ice-cold lysis buffer, and 15 μg of cellular protein from each sample were separated by standard 10% SDS-PAGE and transferred to nitrocellulose membranes. Dilution of both primary and secondary antibodies was made in phosphate-buffered saline containing 0.5% defatted dry milk and 0.1% Tween 20. After 1 h incubation with primary antibody at 1:1000, blots were washed 3 times and an anti-rabbit

secondary peroxidase-conjugated antibody was added for another h. Immunoblots were developed using the Amersham ECL system.

Cell synchronization and cell cycle analysis. U937 cells were synchronized at G_2/M by treating them with 0.05 µg/ml nocodazole for 12 h [32]. The cells were then washed, plated in fresh medium and allowed to progress through the cell cycle. After the indicated times, the cells were washed twice with cold PBS, and fixed with 70% ethanol at 4°C for 18 h. Cells were then washed and resuspended in PBS. RNA was removed by digestion with RNase A at room temperature. Staining was achieved by incubation with staining solution (500 µg/ml propidium iodide in PBS) for 1 h and cell cycle analysis was performed by flow cytometry in a Coulter Epics XL-MCL cytofluorometer.

PC mass quantification. Cell lipids were extracted by the Bligh and Dyer procedure [56], and the individual phospholipid species were fractionated by TLC in silica gel G plates using chloroform/methanol/acetic acid/water (65:501:4) as a solvent system [57]. The PC fraction was identified by comparison with commercial standards. PC mass was quantified by measuring lipid phosphorus [58].

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FIGURE CAPTIONS

Figure 1. *Effect of pyrrophenone on the growth of U937 cells*. A, Dose-response of the effect of pyrrophenone on the Ca^{2+}-dependent PLA_2 activity of U937 cell homogenates. The cell membrane assay was utilized. B, Time-course of the effect of pyrrophenone on the proliferative capacity of U937 cells. The cells were incubated with (closed bars) or without (open bars) 1 μM pyrrophenone for the times indicated, Afterward, cell number was estimated as described under Experimental Procedures. **Data are given as means ± S.E.M. of triplicate determinations, representative of three independent experiments.**

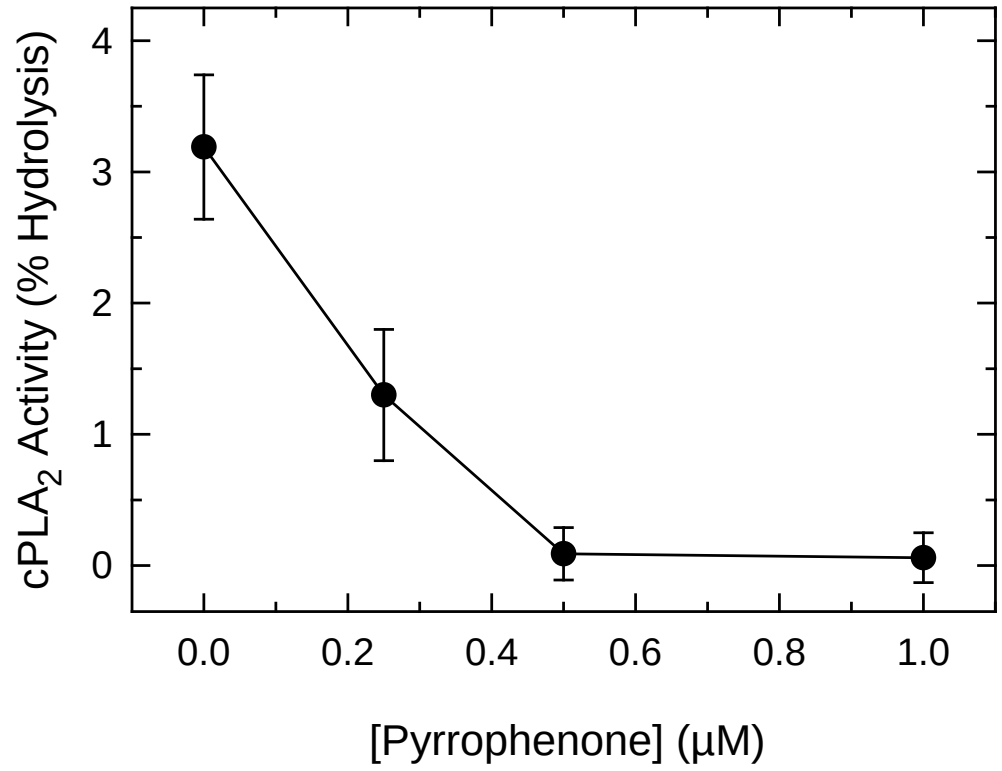
Figure 2. *Effect of BEL on the growth of U937 cells*. A, Dose-response of the effect of BEL on the Ca^{2+}-independent PLA_2 activity of U937 cell homogenates. The substrate was presented in the form of mixed micelles with Triton X-100. B, Time-course of the effect of BEL on the proliferative capacity of U937 cells. The cells were incubated with (closed bars) or without (open bars) 5 μM BEL for the times indicated, Afterward, cell number was estimated as described under Experimental Procedures. **Data are given as means ± S.E.M. of triplicate determinations, representative of three independent experiments.**

Figure 3. *Effect of MAFP on the growth of U937 cells*. A, Dose-response of the effect of MAFP on the Ca^{2+}-independent PLA_2 activity of U937 cell homogenates. The Substrate was presented in the form of mixed micelles with Triton X-100. B, Time-course of the effect of MAFP on the proliferative capacity of U937 cells. The cells were incubated with (closed bars) or without (open bars) 10 μM MAFP for the time indicated, Afterward, cell number was estimated as described under Experimental Procedures. **Data are given as means ± S.E.M. of triplicate determinations, representative of three independent experiments.**

Figure 4. *Antisense inhibition of iPLA$_2$-VIA slows the growth of U937 cells*. The cells were either untreated (inverted triangles), or treated with sense (open circles) or antisense (closed circles) oligonucleotides. Afterward, cell number was estimated as described under Experimental Procedures. Inset shows the iPLA$_2$-VIA protein level after the different treatments (C, control cells, S, sense-treated cells, A, antisense-treated cells), as analyzed by immunoblot. **Data are given as mean ± range of duplicate determinations, representative of five independent experiments.**

Figure 5. *Changes in iPLA$_2$ activity and PC mass during the cell cycle.* The cells were synchronized with nocodazole as described under Experimental Procedures. iPLA$_2$ activity and PC mass were measured at various times after releasing the cells from the nocodazole block, as indicated. **Data are given as mean ± S.E. of triplicate determinations, representative of five independent experiments.**

Figure 6. *Effect of BEL and pyrrophenone on the U937 cell cycle.* The cells were synchronized with nocodazole as described under Experimental Procedures. After releasing the cells from the nocodazole block they were untreated (open symbols) or treated with 5 μM BEL (closed symbols, left column) or 1 μM pyrrophenone (closed symbols, right column), and the percent of cells at various phases of the cell cycle was studied by flow cytometry at different times. **Data are given as mean ± range of duplicate determinations, representative of three independent experiments.**


cPLA2 Activity (% Hydrolysis)
0
1
2
3
4
0.0
0.2
0.4
0.6
0.8
1.0
[Pyrrophenone] (μM)

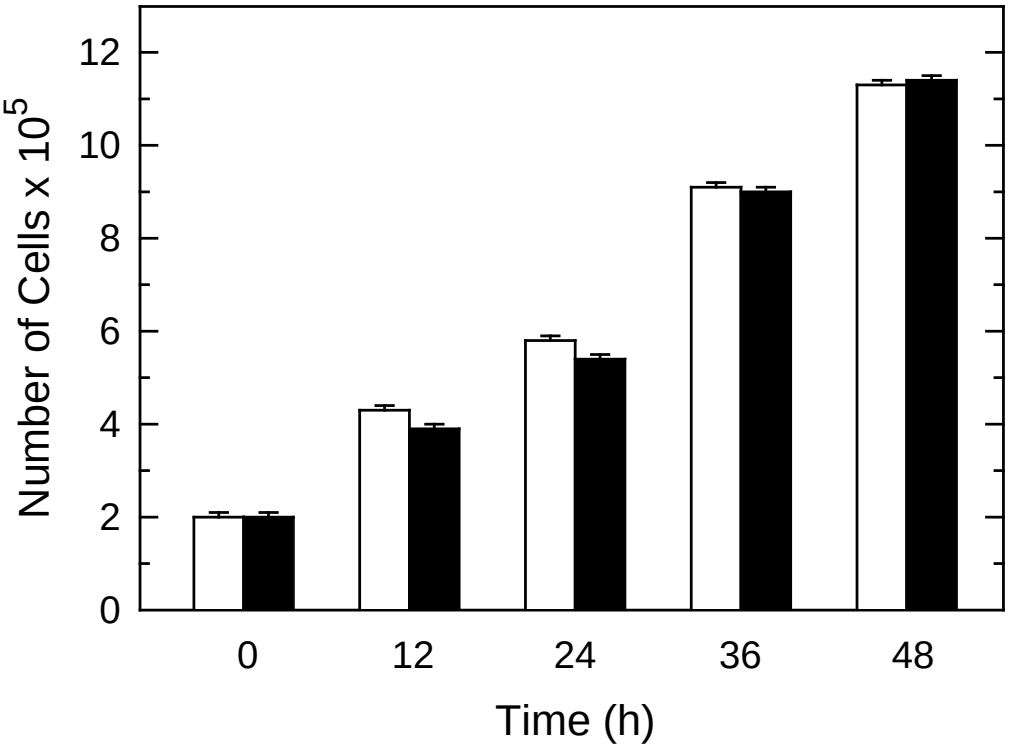
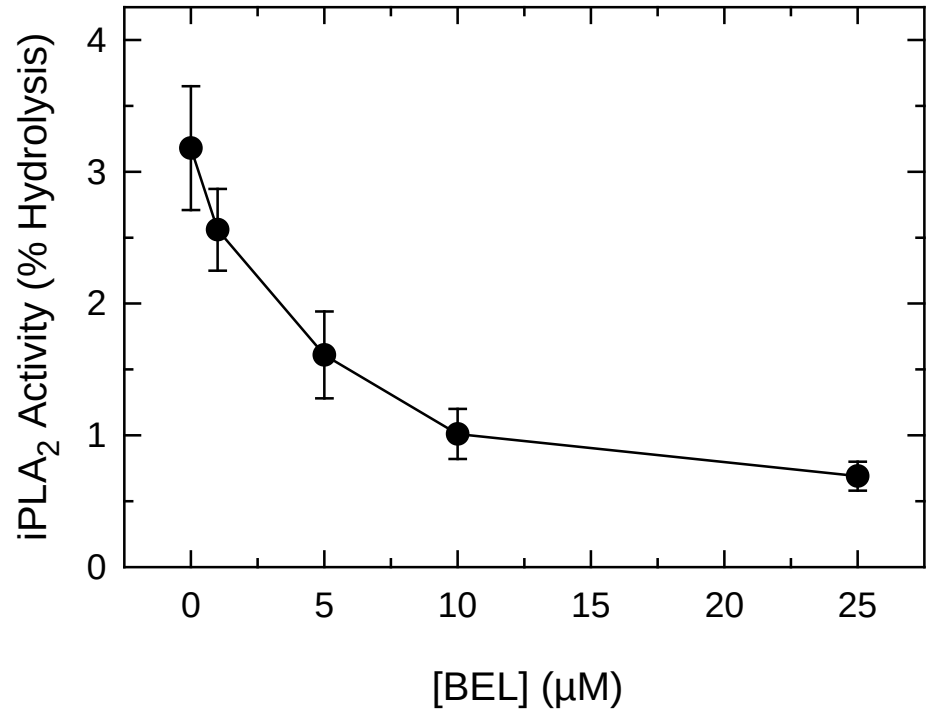

Number of Cells x 10^5
0
2
4
6
8
10
12
0
12
24
36
48
Time (h)

Figure 1


iPLA2 Activity (% Hydrolysis)
0
1
2
3
4
0
5
10
15
20
25
[BEL] (μM)

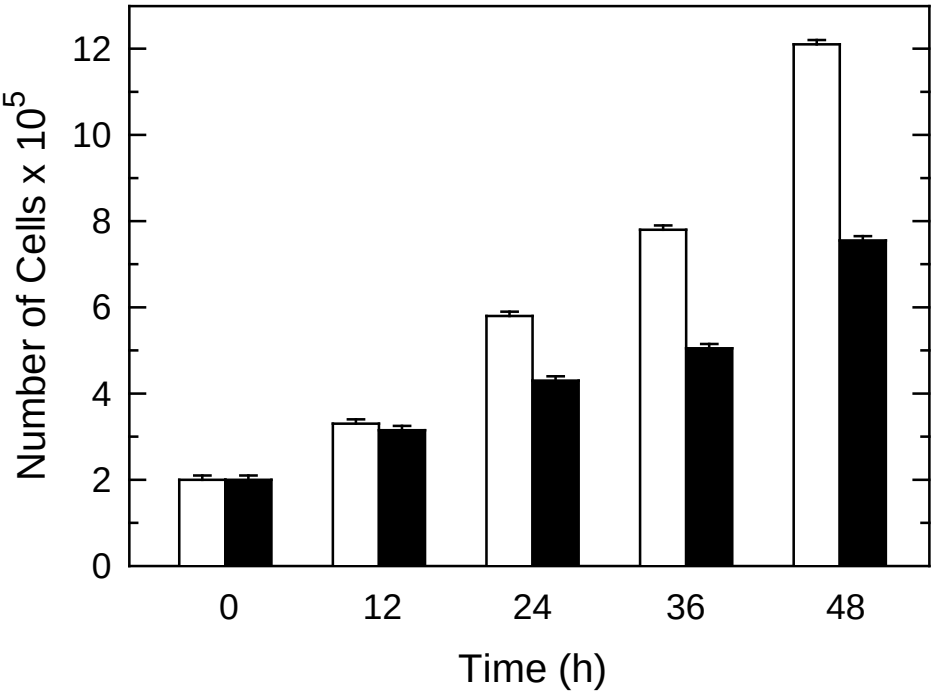
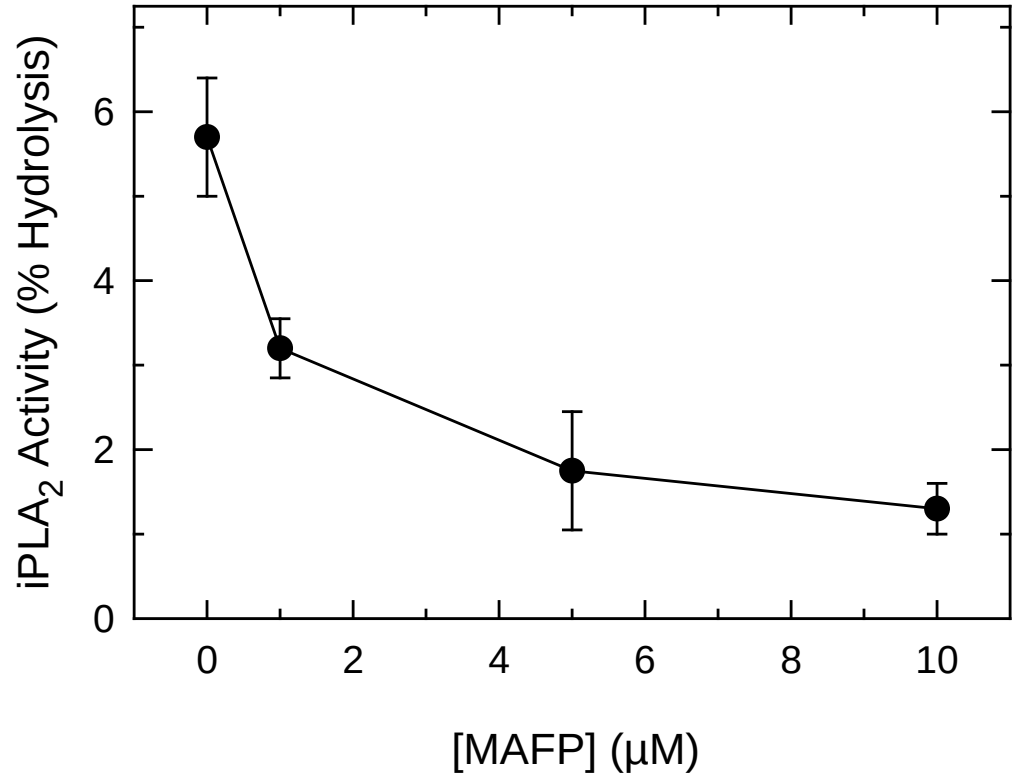

Number of Cells x 10^5
0
2
4
6
8
10
12
0
12
24
36
48
Time (h)

Figure 2


iPLA2 Activity (% Hydrolysis)
0
2
4
6
0
2
4
6
8
10
[MAFP] (μM)

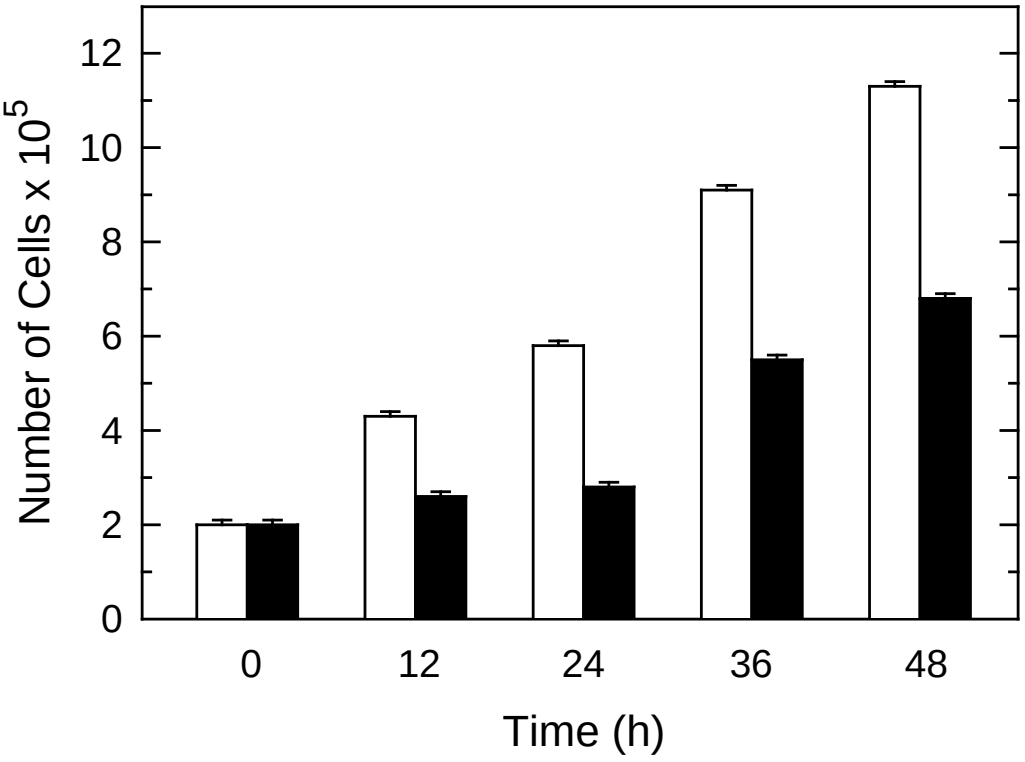

Number of Cells x 10^5
0
2
4
6
8
10
12
0
12
24
36
48
Time (h)

Figure 3

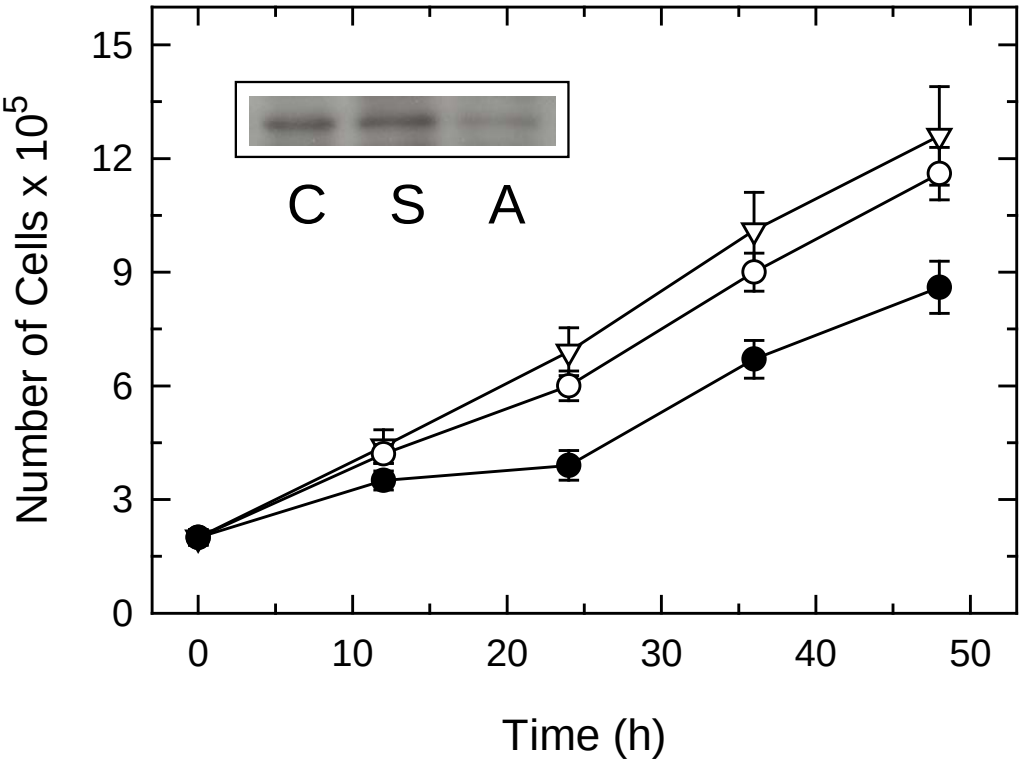


Figure 4

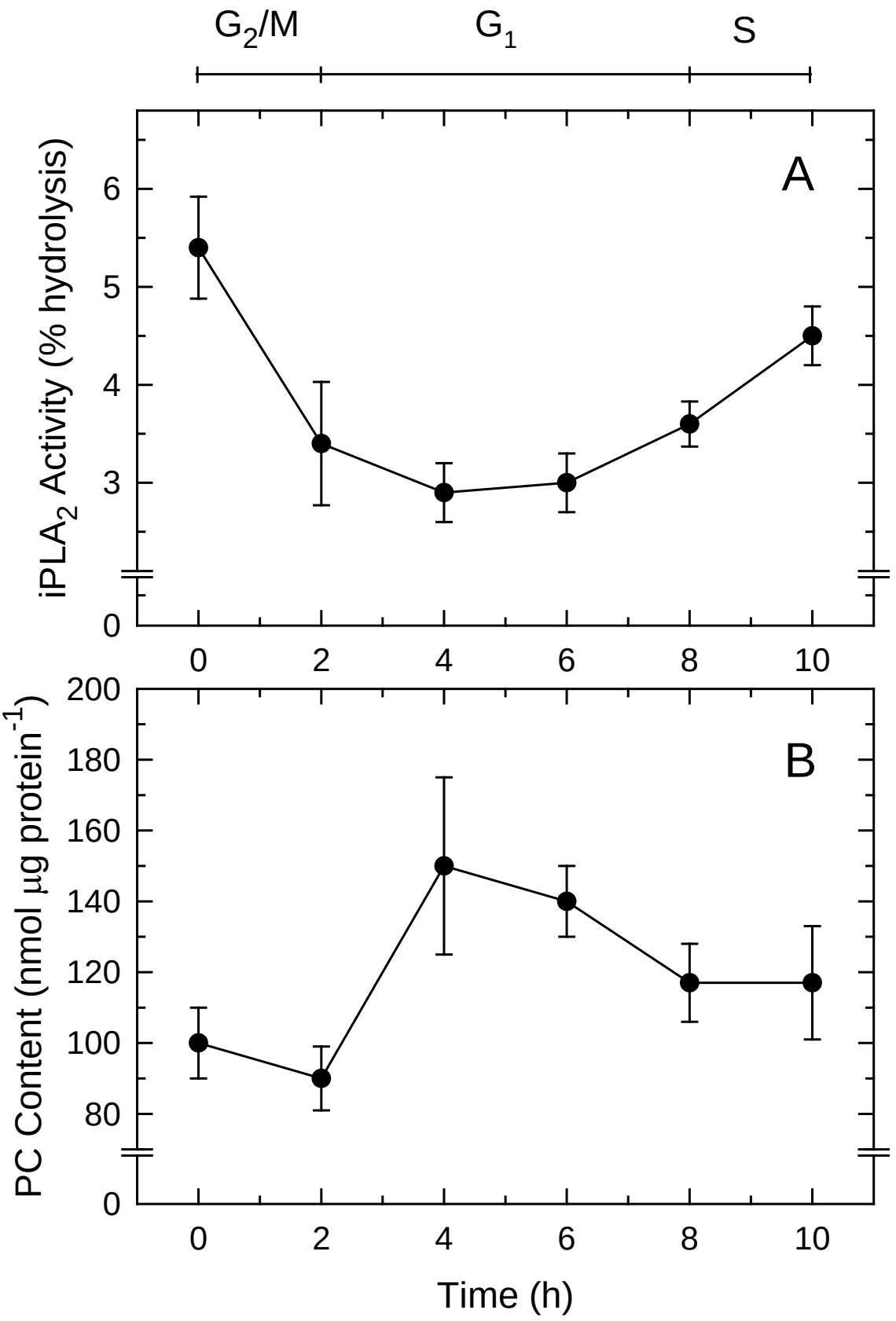

G2/M
G1
S
A
iPLA2 Activity (% hydrolysis)
0
3
4
5
6
0
2
4
6
8
10
B
PC Content (nmol μg protein-1)
0
80
100
120
140
160
180
200
0
2
4
6
8
10
Time (h)

Figure 5

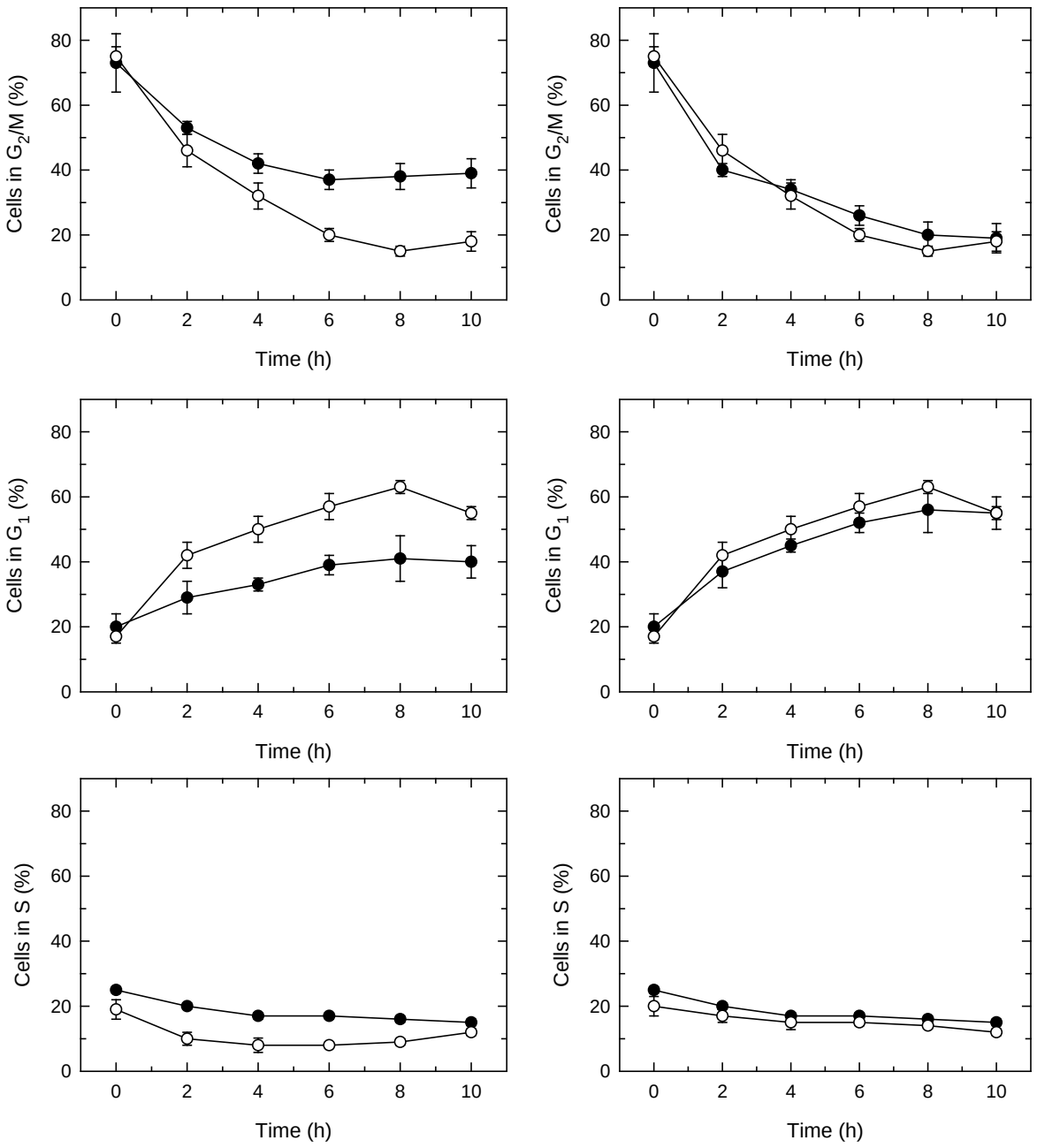

Cells in G2/M (%)
Time (h)
Cells in G1 (%)
Time (h)
Cells in S (%)
Time (h)
0
20
40
60
80
0
2
4
6
8
10

Figure 6